



Full Length Article

Quality Evaluation of *Fritillaria thunbergii* by High-Performance Liquid Chromatography Fingerprinting Coupled with Multicomponent Quantitative Analysis

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Abstract

Medicinal Zhebeimu refers to the dried bulb of the plant *Fritillaria thunbergii*, which belongs to the family Liliaceae. Their main production sites are in Zhejiang, Jiangsu, and Anhui Provinces, with alkaloids as the main components. In this study, according to Chinese Pharmacopoeia, different mobile phases were investigated. Because most alkaloids in *Fritillaria* do not have UV absorption, evaporative light scattering detection (ELSD) was used instead of UV to produce the HPLC-ELSD spectra for five *F. thunbergii* samples and other samples, e.g. *F. chuanxiensis*, *F. anhuiensis*, and *F. ussuriensis*. Comparison showed significant differences exist among these species. There was no significant difference between the *F. thunbergii* samples produced in Zhejiang, which clustered together in both the cluster analyses. However, the *F. thunbergii* samples produced in Nantong, Jiangsu and the *F. thunbergii* samples produced in Zhejiang were separated in the clustering chart, which may be caused by different cultivation environments. The obtained results showed that the combination of quantitative analysis and chromatographic fingerprint analysis can be readily utilized for quality control purposes, offering a comprehensive strategy for quality evaluation of *F. thunbergii* and related products. © 2021 Friends Science Publishers

Keywords: Authenticity analysis; Fingerprint; *Fritillaria thunbergii*; HPLC; Active ingredients; China

Introduction

Chinese medicinal materials are the material basis for the application of traditional Chinese medicine (TCM) in disease prevention and treatment. Their quality directly affects the clinical efficacy of TCM, the experimental research of Chinese herbal medicines, and the quality control of Chinese patent medicines. There are many kinds of Chinese Herbal Medicine (CHM) in China with a wide range of sources, and the treatment by using these medicines vary from place to place. In the market, fake products are often claimed to be genuine, and so-called “superior” products are of a low quality. Such products include not only certified products but also substitutes, fake products, and adulterants with a great discrepancy in quality.

The active ingredients in CHM are mainly secondary metabolites from plants (or animals). These ingredients are influenced by factors, such as the place of production, climate, ecological environment, cultivation or culturing techniques and conditions, harvest seasons, storage conditions, etc., significant differences also occur in the secondary metabolites from the same plant or animal species (Liu *et al.* 2016). In addition, most Chinese

medicinal materials need to be processed and treated before being used as medicine. Different processing and treatment methods also influence the composition of CHM, leading to unstable chemical compositions and quality, which in turn affects their clinical efficacy (Gao *et al.* 2011). Therefore, it is particularly important to find a method that can comprehensively and truly evaluate the quality of CHM.

With an in-depth study on the chemical composition of TCM and the technical improvement of modern instrumental analysis, methods of identification and quality evaluation of TCM are no longer limited to traditional and classic identification measures, such as morphological and microscopic identification. Various modern instruments have been used to perform physical and chemical identifications to reveal the inherent quality of TCM (Qu *et al.* 2014). However, owing to the long-term influence of the quality control mode for chemicals, quality of Chinese medicinal materials is also monitored by measuring the content of an effective or an active ingredient to determine the quality of specific medicinal materials. This mode does not fulfill the requirements of the overall efficacy that TCM advocates and cannot provide a comprehensive or reasonable evaluation of Chinese medicinal materials.

Compound recipes are a significant feature of CHM.

The chemical composition of a single herbal medicine is already quite complex with more than one active ingredient. Sometimes, synergistic effect between the components has a therapeutic role, and often, the antagonistic effect is curative. Therefore, a comprehensive quality evaluation of Chinese medicinal materials should not solely rely on a quantitative determination or qualitative identification of single or several chemical components (Sun et al. 2013). The chromatographic fingerprints of Chinese herbal medicines can provide comprehensive information about their quality and reveal most or even all of the effective components in herbal medicines or all of the active ingredients. This approach is more comprehensive and typical than the detection of the contents of a single component. Combined with other measures, it can be used to determine the quality and authenticity of Chinese medicinal materials.

Fritillaria refers to the bulb of many plants that belong to the genus *Fritillaria* L. in the family Liliaceae. Chinese Pharmacopoeia (2020) includes nine species of *Fritillaria*, which are *F. thunbergii*, *F. cirrhosa*, *F. unibracteata*, *F. przewalskii*, *F. delavayi*, *F. ussuriensis*, *F. pallidiflora*, *F. walujewii* and *F. hupehensis*. Among these species, the original plants of the medicinal material of *F. chuanxiensis* have been determined to be *F. cirrhosa*, *F. unibracteata*, *F. przewalskii* or *F. delavayi*; and the original plants of Siberian fritillary are *F. pallidiflora* or *F. walujewii*. Meanwhile, *F. chuanxiensis* and Zhejiang fritillary are both dominant products in the domestic medicinal materials market in China (Shan et al. 2017). *Fritillaria* is widely distributed with complex germplasm resources. People from various localities have different customs in the application of these materials. In addition, the prices of *Fritillaria* vary greatly based on the species and origins. Therefore, to ensure the efficacy and safety of this medical treatment, it is important to carry out research on the sources of this medicinal material.

The Bulbus *F. thunbergii* in the group of "eight herbal medicines of Zhejiang" is a famous Chinese medicinal material in Zhejiang Province (Yuan et al. 2010). It is of high quality and stable texture, and the associated products are sold in many places within and outside the province and further to countries and regions in Southeast Asia. This material is large with a shape of Yuanbao (gold or silver ingots with a Chinese style); thus, it is also called Yuanbao fritillary. Wild *F. thunbergii* is distributed in southern Jiangsu, northern Zhejiang, Hunan, etc. Large-scale cultivations occur in Ningbo, Pan'an of Zhejiang Province, and Nantong in Jiangsu Province, and it is also cultivated in other provinces, such as Hunan, Hubei, Sichuan, Anhui, and Henan. However, besides the cultivation bases, majority of medicinal materials are purchased from individual farmers. Problems, such as unclear sources and mixed varieties, often occur. To ascertain the authenticity of medicinal materials, to establish quality standards for the varieties, and to guide

Good Agricultural Practices (GAP) production, we conducted a chemical fingerprint study of *F. thunbergii*.

Thus far, more than 130 compounds have been isolated and identified from *Fritillaria* plants. These compounds are mainly alkaloids, most of which are isosteroidal alkaloids, and only a few are steroidal alkaloids or other types of alkaloids (Temel et al. 2015; Smolskait et al. 2015; Wang et al. 2018). Based on the heterocyclic structures of these compounds, isosteroid alkaloids from *Fritillaria* can be divided into the cevanine, jervine, and veratramine groups (Lyu et al. 2015; Ruan et al. 2016; Wang et al. 2018). There are also 15 steroidal alkaloids and one other alkaloid (Arshadi et al. 2012; Temel et al. 2015).

Currently, methods for the identification of *Fritillaria* include high-performance liquid fingerprint chromatography (HPLC), thin-layer chromatography, infrared, and ultraviolet spectroscopies, etc. Recently, there have been many research studies on HPLC fingerprinting. Zhang et al. (2015) established a fingerprint analytical method for *F. hupehensis* using HPLC-ELSD using the experimental details as follows: the column was a Hypersil ODS (250 × 4.6 mm), mobile phase was methanol (containing 0.05% triethylamine)–water for gradient elution, flow rate was 1.0 mL/min, and detector was an ELSD. Li et al. (2006) examined 11 *Fritillaria* samples using HPLC according to the following details: XDP-C18 (4.6 × 250 mm) as the chromatographic column, the mobile phase was methanol–water in the ratio of 70:30 (v/v) containing 7.5 mmol/L sodium dodecyl sulfate (SDS) with the pH at 4.5 ± 0.1, and the detection wavelength was 215 nm. Based on the retention time and relative peak area of different chemical components in the sample, chemometric methods were applied to calculate and cluster the important parameters used to describe the degree of separation and quality of two adjacent chromatographic peaks. The results revealed the consistency between the cluster analysis and the botanical classification. This method can be used for the identification of *Fritillaria*, and it aided the establishment of the characteristic fingerprints of *Fritillaria*. Chen et al. (2013) used HPLC–differential thermal analysis (DTA) to determine the quality of *F. thunbergii*, *F. thunbergii* var. *chekiangensis*, *F. hupehensis*, and *F. anhuiensis* obtained under the same ecological environments with *Fritillaria* origins and different processing methods. These studies showed that *Fritillaria* samples processed by traditional methods contained the same ingredients and can be mixed, whereas samples fumigated with sulfur were different owing to the presence of sulfur dioxide; additionally, the *Fritillaria* dried with lime was stable (Mittra et al. 2009). Wang et al. (2017) reported that the alkaloids of *Fritillaria* from Jilin indicated significant difference in the HPLC spectra of extracts using different solvents and different methods. Lim et al. (2018) established an HPLC-ELSD method for the detection of *Fritillaria* alkaloids and gluco-alkaloid to provide an accurate and concise approach for quality control of *Fritillaria* medicinal materials.

In addition to HPLC fingerprinting, many researchers also use thin-layer chromatography to identify *Fritillaria* medicinal materials. Wu *et al.* (2010) applied thin-layer chromatography to compare *F. ussuriensis* of different origins and harvesting periods with different varieties of *Fritillaria* and counterfeits, and this method provided a reliable basis for the identification of the medicinal materials of *F. ussuriensis*. Li *et al.* (2006) used microscopic examination and TLC to identify *F. pallidiflora* with *F. walujewii* as reference substances. Rashid *et al.* (2015) identified and differentiated fresh tubers of *F. thunbergii* and its adulterant with TLC and ultraviolet spectral identification to provide a basis for further identification. Huang *et al.* (2005) analyzed the total saponins from various *Fritillaria* medicinal materials using thin-layer chromatography to distinguish *F. chuanxiensis* from other fritillary materials.

There have been also many studies on the identification of *Fritillaria* using spectroscopic techniques. Lee *et al.* (2015) combined near-infrared diffuse reflectance spectroscopy and principal component analysis (PCA) to show that this approach can identify *Fritillaria* and its related species, being capable of clustering and identification. Mitra *et al.* (2009) established a method using near-infrared diffuse reflectance spectroscopy for the identification of *Fritillaria* and its counterfeits, providing a new method for the identification and analysis of *Fritillaria* and other TCM. Smolskait *et al.* (2015) used FTIR spectroscopy and cluster analysis to identify and analyze *Fritillaria* medicinal materials and their counterfeits. Li *et al.* (2014) applied FTIR spectroscopy to directly determine the FTIR spectra of TCM, which provided a direct, quick, and accurate measurement of *F. chuanxiensis*, *F. thunbergii*, and *F. thunbergii* var. *chekiangensis*. Majo *et al.* (2011) used UV spectrophotometry to measure and compare the UV spectra of anhydrous ethanol extracts of *F. walujewii* and four other types of *Fritillaria* samples. The results revealed that *F. walujewii* showed consistent differences from the other four types of *Fritillaria*.

Although there are many studies on the chemistry and fingerprints of *F. thunbergii*, the fingerprints of medicinal materials produced in Pan'an County, which is the main production site of medicinal materials in Zhejiang Province, have not been studied previously. This study focused on the chemical fingerprinting of medicinal materials of *F. thunbergii* produced in Pan'an to establish quality control standards for the medicinal materials produced in Pan'an County and to guide GAP production.

Materials and Methods

Test materials

The test materials were *F. thunbergii* samples collected from Pan'an, Ningbo of Zhejiang Province and Nantong of Jiangsu Province, *F. chuanxiensis* from Maoxian, Sichuan

Province, and *F. ussuriensis* from Jilin Province during the spring and summer time of 2018~2019 (Table 1). Among them, there are two varieties of *F. chuanxiensis*: one has a shorter growth period and a smaller bulb, and the other has a longer growth period and a larger bulb.

Preparation of test solutions

According to the Chinese Pharmacopoeia (2020 edition), the extraction method of *F. thunbergii* was slightly modified to prepare the test solution. A 2 g of the test powder were accurately weighed, placed in an Erlenmeyer flask, and immersed in 4 mL of concentrated ammonia solution for 1 h. Forty mL of a chloroform-methanol (4:1) solvent was added, weighed, mixed well, and the flask was placed in a 40 °C water bath for 30 min and filtered. The filtrate was evaporated to dryness, and the residue was dissolved in methanol (chromatography grade) and transferred to a 10 mL volumetric flask. Methanol was added to make up the final volume, and the solution was filtered through a 0.45 µm filter membrane.

Preparation of the reference solution

Peimine and peiminine standards were accurately weighed and dissolved in methanol during ultrasonication to make a 0.1 mg/mL reference solution.

Setup of chromatographic conditions

As described by Wei *et al.* (2017), methanol-water (0.03% triethylamine) was used as the mobile phase for gradient elution. The experimental results were promising. The following gradient elution procedure was set up through repeated experiments: flow rate of 1 mL/min, injection volume of 5 µL. The ELSD detection parameters were as follows: drift tube temperature of 35°C and carrier gas flow rate of 2.2 L/min.

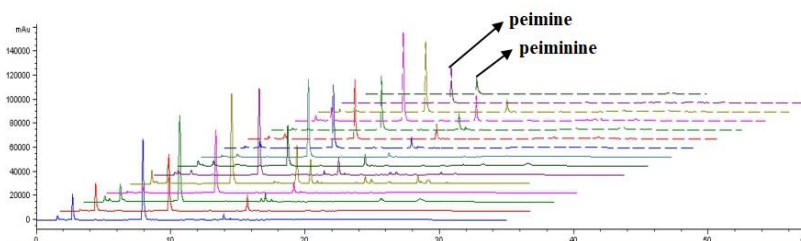
Data analysis

Similarity analysis of fingerprints: Fingerprints were exported in AIA format from the Agilent chromatographic workstation, and imported into the 'Chinese Herbal Medicine Chromatographic Fingerprint Similarity Evaluation System' (promulgated by the National Pharmacopoeia Commission, Version A) software. Multiple operation steps were performed to obtain the analytical results, including trimming of the first 5 min of the chromatograms, baseline adjustment, multi-point correction, automatic matching, and similarity analysis after generating the control spectra.

Cluster analysis: Peaks in the fingerprint spectra were automatically integrated using the Agilent chromatographic workstation to obtain the area of each peak and to calculate the relative peak area by dividing each peak by the area of

Table 1: Sample of regular table design Details of plant materials collected from different regions

No.	Name	Origin	Time
S1	<i>Fritillaria cirrhosa</i> (small)	Songpinggou, Maoxian County, Sichu	Jun. 2018
S2	<i>F. cirrhosa</i> (large)	Songpinggou, Maoxian County, Sichu	Jun. 2018
S3	<i>F. thunbergii</i> var. <i>chekiangensis</i>	Yanshang village, Pan'an, Zhejiang	Jun. 2018
S4	<i>F. ussuriensis</i>	Huinan County, Tonghua City, Jilin Province	Aug. 2018
S5	<i>F. anhuiensis</i>	Cixia village, Pan'an, Zhejiang	Jun. 2018
S6	<i>F. anhuiensis</i>	Yanshang village, Pan'an, Zhejiang	Jun. 2018
S7	<i>F. thunbergii</i>	Zhangzhishan, Nantong, Jiangsu	Jun. 2018
S8	<i>F. thunbergii</i> (many-seeded)	Houtang village, Pan'an, Zhejiang	Jun. 2018
S9	<i>F. thunbergii</i>	Zhangshui, Ningbo, Zhejiang	Jun. 2018
S10	<i>F. thunbergii</i>	Cixia village, Pan'an, Zhejiang	Jun. 2018
S11	<i>F. thunbergii</i>	Houtang village, Pan'an, Zhejiang	Jun. 2018
S12	<i>F. thunbergii</i>	Pantanlengshui, Pan'an, Zhejiang	Jun. 2018
S13	<i>F. thunbergii</i>	Yangzhai village, Pan'an, Zhejiang	Jun. 2018

**Fig. 1:** HPLC-ELSD fingerprints of 13 *Fritillaria* samples

the corresponding peaks of peimine and peiminine. An absent peak was assigned a peak area of 0. The relative peak area was used as the basis for clustering analysis. The cluster analysis function in the multivariate analysis menu in the DPS system was used to standardize the original data, based on Euclidean distance measurement (Saurabh *et al.* 2017) and shortest-distance clustering, DPS V8.01.

Principal component analysis (PCA): The relative peak area of each peak in the fingerprints was obtained in the same way as the cluster analysis. Based on the relative peak area, the PCA analysis function, which is an unsupervised multivariate data analysis approach, in the multivariate analysis menu in the DPS V8.01 was used to analyze the original data to obtain the PCA chart.

Calibration of chromatographic peaks in the HPLC-ELSD fingerprints: A 5 μ L of the standard solutions of peimine and peiminine were injected into a HPLC and measured according to the determined chromatographic conditions to produce the chromatograms (Fig. 1).

Investigation of fingerprinting methodology

After determination of the extraction and chromatographic separation conditions for the samples, we conducted a methodological investigation on the effectiveness of the entire method. Investigation indicators were selected from the following three aspects: the precision of the instruments, stability of the samples, and reproducibility of the method. Details are described as follows.

Precision test: A test solution of *Fritillaria* sample 6 was precisely injected at a volume of 5 μ L five consecutive

times. The chromatogram was recorded, and the relative retention time, relative peak area, and RSD of each peak were calculated. The results showed that the RSD% values of the relative retention time of the common peaks were between 0.18% and 3.01%, and the RSD% values of the relative peak area were between 2.1% and 3.46%. ELSD detection has lower precision than UV detection, but it basically meets the technical requirements of fingerprints, controlled within 5%.

Stability test: A test solution of *Fritillaria* sample 5 was precisely injected at a volume of 5 μ L and at intervals of every 2, 4, 6, 12, and 24 h. The results revealed that the RSD% values of the relative retention time of the common peaks were from 1.6 to 2.84, which were all less than 3%. The RSD% values of the relative peak area were between 1.9% and 4.23%, which indicated that the tested product was stable within 24 h.

Repeatability test: Five replicates of *Fritillaria* sample 6 were prepared as the test solution according to the determined method. The solutions were injected separately, and the chromatograms were recorded. The results showed that the RSD% values of the relative retention time of the common peaks were between 0.97% and 1.35%, which were all less than 3%. RSD% values of the relative peak area were between 2.74% and 4.26%. Fig. 1 shows that the retention times of peimine and peiminine standards were 8.102 and 8.252 min, respectively, and the time difference between the two was only 0.15 min. The retention times of peimine and peiminine in the test solutions were almost the same, showing a large peak at approximately 8 min in the fingerprints.

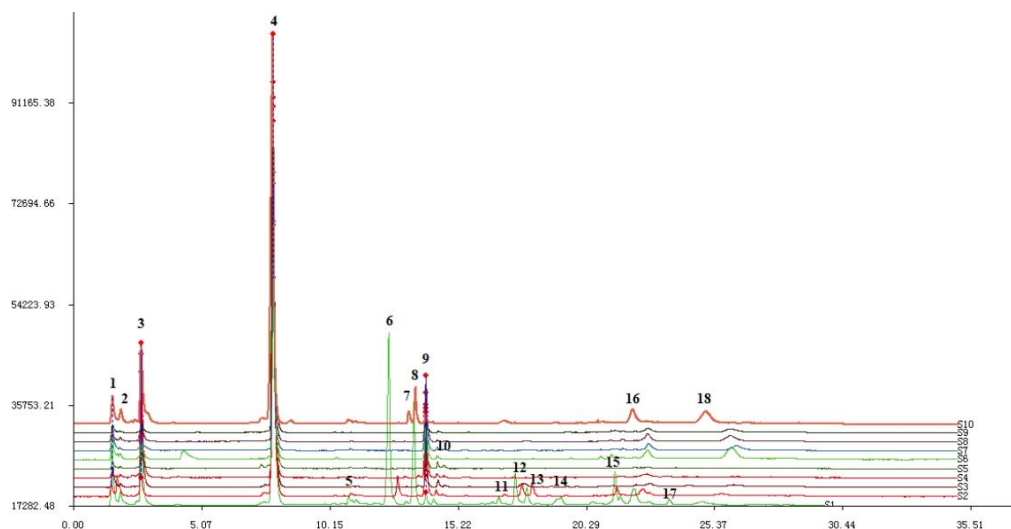


Fig. 2: Compare of different *Fritillaria* species (after calibration)

Results

Analysis and comparison of HPLC fingerprints of different species of *Fritillaria*

In this study, the HPLC spectra of *F. thunbergii*, *F. thunbergii* var. *chekiangensis*, *F. anhuiensis*, *F. chuanxiensis*, and *F. ussuriensis* were compared. Fig. 2 shows that the spectra of different species of *Fritillaria* are quite different. The chromatograms were imported into the "Chinese Herbal Medicine Chromatographic Fingerprint Similarity Evaluation System" in AIA format with multi-point correction. *F. anhuiensis* had the highest number of peaks. Although *F. thunbergii* var. *chekiangensis* is a variant of *F. thunbergii*, its chemical composition was significantly different from *F. thunbergii* with an extra peak (No. 7) compared to other *Fritillaria*, and the retention time of each peak was quite different from that of other samples. The HPLC spectrum of multi-seed *F. thunbergii* was similar to that of *F. thunbergii*, but its content is not as high as that of *F. thunbergii*. Number 10 peak was present in the spectra of *F. ussuriensis*, *F. chuanxiensis*, and *F. anhuiensis* but not in the spectra of *F. thunbergii* and *F. thunbergii* var. *chekiangensis*.

HPLC-ELSD fingerprint similarity of 13 batches of *Fritillaria*

Similarity analysis was carried out using the method specified previously. The results showed that the similarities among the *F. thunbergii* samples were all above 0.90. The similarities among *F. chuanxiensis* (large), *F. anhuiensis* and *F. thunbergii* were between 0.80 and 0.90, indicating that they can be distinguished. However, the similarity between *F. ussuriensis* and *F. thunbergii* was also above

0.90, implying the difficulty in distinguishing these two species.

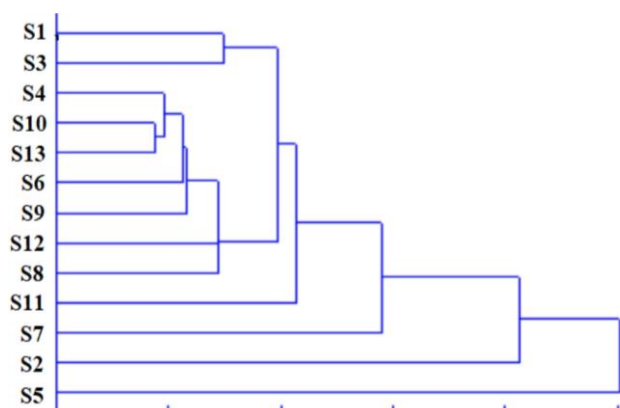
Cluster analysis of 13 batches of *Fritillaria*

Different *Fritillaria* samples were considered as one entity. By comparing the properties and characteristics of the relative peak area change among the different samples, i.e., the areas of all peaks were divided by the peak area of the No. 4 peak, the relative peak areas of the 13 *Fritillaria* samples were systematically clustered by the DPS system (Table 2). The Euclidean distance was used for the measurement, and clustering was done using the minimum distance method (Fig. 3).

Results showed that the *F. anhuiensis* product grown in Cixia village of Pan'an showed the most significant difference from other *Fritillaria* species, followed by *F. chuanxiensis* with a longer growth period (Fig. 4). *F. thunbergii* samples grown in Nantong of Jiangsu were not clustered together with the samples of the same species grown in Zhejiang, indicating a difference between the chemical compositions of the *F. thunbergii* samples from these two sources, which may be influenced by the growing environment. Although *F. thunbergii* var. *chekiangensis* is a variety of *F. thunbergii*, its chemical composition was significantly different from that of *F. thunbergii*. The difference was evident in the fingerprints as follows: peaks 2 and 7 are absent from the spectrum of *F. thunbergii*, and they cluster with *F. chuanxiensis*, which has a short growth period. *F. ussuriensis* and the *F. thunbergii* produced in Cixia and Yangzhai clustered together. However, according to the HPLC spectra, *F. anhuiensis* and *F. thunbergii* can be easily distinguished. It is likely that the source of *F. anhuiensis* was different. The fingerprints of *F. ussuriensis* and *F. thunbergii* were very similar, and *F. ussuriensis*

Table 2: Similarity of HPLC fingerprints from 13 *Fritillaria* sample

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13
S1		0.856	0.95	0.965	0.872	0.963	0.902	0.979	0.942	0.974	0.927	0.977	0.979
S2	0.856		0.862	0.928	0.816	0.891	0.914	0.878	0.931	0.892	0.893	0.891	0.893
S3	0.95	0.862		0.97	0.908	0.972	0.903	0.975	0.944	0.956	0.933	0.954	0.967
S4	0.965	0.928	0.970		0.902	0.992	0.945	0.99	0.984	0.983	0.966	0.981	0.99
S5	0.872	0.816	0.908	0.902		0.921	0.857	0.894	0.893	0.900	0.886	0.9	0.903
S6	0.963	0.891	0.972	0.992	0.921		0.936	0.988	0.978	0.985	0.966	0.984	0.991
S7	0.902	0.914	0.903	0.945	0.857	0.936		0.931	0.959	0.958	0.976	0.936	0.95
S8	0.979	0.878	0.975	0.99	0.894	0.988	0.931		0.965	0.982	0.957	0.975	0.992
S9	0.942	0.931	0.944	0.984	0.893	0.978	0.959	0.965		0.981	0.97	0.978	0.98
S10	0.974	0.892	0.956	0.983	0.900	0.985	0.958	0.982	0.981		0.984	0.994	0.998
S11	0.927	0.893	0.933	0.966	0.886	0.966	0.976	0.957	0.97	0.984		0.967	0.978
S12	0.977	0.891	0.954	0.981	0.900	0.984	0.936	0.975	0.978	0.994	0.967		0.992
S13	0.979	0.893	0.967	0.99	0.903	0.991	0.95	0.992	0.98	0.998	0.978	0.992	

**Fig. 3:** Result of cluster analysis for *Fritillaria* species

showed an extra peak (No. 9) compared with the spectrum of *F. thunbergii*.

PCA of 13 batches of *Fritillaria*

In addition to the cluster analysis of the HPLC fingerprints of *Fritillaria*, we also applied PCA to analyze the data of all *Fritillaria* samples (Fig. 5). It was concluded that samples S7 and S5 were quite different from other samples; *F. thunbergii* samples produced in Nantong of Jiangsu and *F. anhuiensis* samples produced in Cixia of Pan'an, showed different HPLC fingerprints from other *Fritillaria* samples. This indicated that there was a difference in the composition or content between the *F. thunbergii* samples produced in Jiangsu and Zhejiang Province, which further confirmed the authenticity of these medicinal materials. Almost all other samples clustered together, and they could not be compared. However, the PCA results can be combined with the cluster analysis results to reveal information as follows. Three samples with the greatest differences were *F. anhuiensis* from Cixia Village of Pan'an, *F. thunbergii* from Nantong of Jiangsu, and *F. chuanxiensis* with a longer growth period. S2, S3, S4 and S6 were distributed in the sample region with all other *F. thunbergii* samples, but were not distinguished in this analysis.

Discussion

The HPLC fingerprints of different species of *Fritillaria* were analyzed by HPLC-ELSD and further compared. The results showed that the greatest difference was between *F. anhuiensis* from Cixia Village of Pan'an and other *Fritillaria* samples. Under the same extraction and detection conditions, *F. anhuiensis* have more fingerprint peaks than those of *F. thunbergii*, *F. chuanxiensis* and *F. ussuriensis*. *F. anhuiensis* samples are clustered together with *F. thunbergii* in the clustering charts, but their HPLC spectra indicate that some peaks of the two types of *F. anhuiensis* samples are consistent, such as peak No. 6, 12, 13, and 15, all of which are unique to *F. anhuiensis* (Fig. 3). Further investigation is needed to determine the causes of this difference. One possible reason is that local farmers introduced seeds from different sources. The other is that these two *F. anhuiensis* samples may belong to different species. In addition, *F. anhuiensis* is just a vague collective term for *Fritillaria* species in Anhui. There are many specific varieties, such as Anhui *Fritillaria*, Wanjiang *Fritillaria*, Wannan *Fritillaria*, and Wanxi *Fritillaria* (Paz et al. 2015). Second, *F. chuanxiensis* with a longer growth period is isolated, whereas *F. chuanxiensis* with a shorter growth period was gathered first with *F. thunbergii* var. *chekiangensis* and then with *F. thunbergii*, indicating that the chemical composition of *Fritillaria* is a dynamic accumulation process. The chemical composition of *Fritillaria* with different growth years may not be identical, which provides a basis for the harvest time limits of medicinal materials. *F. thunbergii* var. *chekiangensis* is a variety of *F. thunbergii*. It is mainly produced in Pan'an, Zhejiang. It has a smaller size, similar to *F. chuanxiensis*. It has been used to replace *F. chuanxiensis* by the public. However, its chemical composition is closer to that of *F. thunbergii* with some differences. Zhao et al. (2014) analyzed its chemical composition and confirmed that it is a variety of *F. thunbergii*. It is quite different from *F. chuanxiensis* and cannot replace it. Of the *F. thunbergii* samples, there was no obvious difference between the *F. thunbergii* collected in Zhejiang Province (Pan'an and Ningbo), but the *F. thunbergii* from Nantong, Jiangsu was

isolated separately, indicating that there were differences in *F. thunbergii* from different regions. This may have been caused by the growing environment, and it further confirmed the authenticity of the medicinal materials.

In this study, *F. ussuriensis* and *F. thunbergii* were clustered together. A review of relevant studies showed that the main alkaloid components in *F. ussuriensis* are pingpeimine A and pingpeimine B (Sun *et al.* 2013), which have similar chemical structures as peimine and peiminine. Thus, they may have retention times that are very close to those of *F. thunbergii* in the HPLC fingerprints. *F. ussuriensis* also contains peimine at a level of 0.002–0.003% (Chen *et al.* 2011), which may coelute with pingpeimine A and pingpeimine B to form a huge peak at approximately 8 min. The above reasons caused their fingerprints to be very similar in this study and not distinguishable.

Based on the HPLC-ELSD fingerprints of 13 batches of *Fritillaria*, five types, i.e., *F. thunbergii*, *F. thunbergii* var. *chekiangensis*, *F. chuanxiensis*, *F. anhuiensis* and *F. ussuriensis*, were compared to obtain preliminary results. However, there exist some problems and issues. 1) there were only 13 batches of samples with only eight batches of *F. thunbergii* samples (seven taken within the province and one taken outside the province). To establish the HPLC fingerprints of *F. thunbergii* from Zhejiang Province, at least 10 batches of samples from places within the province were required. Thus, the number of samples needs to be increased; 2) information on the samples was partially missing, for example, the purchased *F. anhuiensis* sample did not have a specific species name and 3) the HPLC detection conditions need to be further optimized.

Conclusion

HPLC-ELSD fingerprints could well distinguish *F. thunbergii* grown in Pan'an, and *F. thunbergii* var. *chekiangensis*, *F. anhuiensis*, *F. thunbergii*, *F. chuanxiensis*, and *F. ussuriensis* samples from places outside Zhejiang Province, indicating that the environment has an important influence on the composition of medicinal materials, which confirmed the authenticity of the medicinal materials. In this study, only the fingerprints of the alkaloids of *Fritillaria* were studied. The non-alkaloids were excluded because many of the components in *Fritillaria* are similar in structure, only differing in some of the substituent groups. This makes it difficult to distinguish these components in the HPLC spectra, and more investigation is needed to optimize the HPLC separation conditions.

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Author Contributions

ZJ conceived the idea of work; QM conducted formal analysis; RH investigated the materials; RH and QM wrote the original draft; YL and JZ reviewed and edit the draft. All authors have read and agreed to the published version of the manuscript.

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